

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
18 April 2002 (18.04.2002)

PCT

(10) International Publication Number  
**WO 02/30207 A1**

- (51) International Patent Classification<sup>7</sup>: **A21D 8/04**
- (21) International Application Number: PCT/DK01/00646
- (22) International Filing Date: 4 October 2001 (04.10.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
PA 2000 01521 12 October 2000 (12.10.2000) DK  
60/241,139 17 October 2000 (17.10.2000) US
- (71) Applicant (for all designated States except US):  
NOVOZYMES A/S [DK/DK]; Krogshøjvej 36, DK-2880  
Bagsværd (DK).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): CHRISTIANSEN,  
Luise [DK/DK]; Sommerstedgade 11, 2.th., DK-1718  
København V (DK). BUDOLFSEN, Gitte [DK/DK];  
Drosselvej 53M, DK-2000 Frederiksberg (DK).
- (74) Common Representative: NOVOZYMES A/S; Patents,  
Krogshøjvej 36, DK-2880 Bagsværd (DK).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI,  
SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU,  
ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,  
CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,  
TG).
- Published:  
— with international search report  
— before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments
- For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.



**WO 02/30207 A1**

(54) Title: PREPARATION OF BAKED PRODUCT FROM DOUGH

(57) Abstract: Addition of an oxidase and a protein disulfide isomerase (PDI) to a dough has a syn-ergistic effect on the loaf volume of a baked product made from the dough.

## PREPARATION OF BAKED PRODUCT FROM DOUGH

### FIELD OF THE INVENTION

The present invention relates to a process for preparing a baked product made from dough. More particularly, it relates to a process for preparing a baked product with an increased loaf volume.

### BACKGROUND OF THE INVENTION

In the preparation of bread and other baked products from dough, it is generally desirable to increase the volume of the baked product.

The prior art discloses the addition to dough of various oxidases which act on a substrate in the dough to form hydrogen peroxide, e.g. to obtain a larger loaf volume. A well known example is glucose oxidase from *Aspergillus niger* which acts on glucose, maltose etc. in the dough.

WO 9500636 discloses the use of a protein disulfide isomerase for strengthening of gluten in bakery or pastry products.

WO 9529996 and WO 9931990 disclose the addition to dough of certain oxidases in combination with a protein disulfide isomerase.

### SUMMARY OF THE INVENTION

The inventors have found that the addition of an oxidase and a protein disulfide isomerase (PDI) to a dough has a synergistic effect on the loaf volume of a baked product made from the dough.

Accordingly, the invention provides a process of preparing a baked product by adding a hydrogen peroxide-forming oxidase and a protein disulfide isomerase to a dough, and baking the dough. The oxidase is a hydrogen peroxide-forming oxidase except the following:

a) a glucose oxidase having optimum activity at pH 6-7 and having more than 75 % of maximum activity at pH 8, and

b) a carbohydrate oxidase which has a higher activity on an oligosaccharide having a degree of polymerization of 2 or higher as a substrate than on the corresponding monosaccharide.

The invention also provides a baking composition for use in the process.

## DETAILED DESCRIPTION OF THE INVENTION

### Hydrogen peroxide-forming oxidase

The following are some examples of oxidases with EC numbers (according to Enzyme Nomenclature): A monosaccharide oxidase such as glucose oxidase (EC 1.1.3.4), hexose oxidase (EC 1.1.3.5), galactose oxidase (EC 1.1.3.9) or pyranose oxidase (EC 1.1.3.10). A deaminating oxidase such as L-amino acid oxidase (EC 1.4.3.2), amine oxidase (EC 1.4.3.4).

The oxidase may particularly have optimum activity at pH 4 - 5.5.

The glucose oxidase may be derived from a strain of *Aspergillus* or *Penicillium*, particularly *A. niger*, *P. notatum*, *P. amagasakiense* or *P. vitale*. The hexose oxidase may be one described in EP 833563. The pyranose oxidase may be one described in WO 9722257, e.g. derived from *Trametes*, particularly *T. hirsuta*. The galactose oxidase may be one described in WO 0050606.

The deaminating oxidase may be one described in WO 9721351, e.g. a benzylamine oxidase derived from *Pichia*, particularly *P. pastoris*.

The glucose oxidase will typically be added in an amount corresponding to 20-1,000 GODU/kg flour, particularly 75-300 GODU/kg (GODU activity unit defined below). For other oxidases, a similar assay may be used by substituting the substrate.

A substrate of the oxidase may already be present in the dough (e.g. glucose or maltose), or it may be added.

### 20 Assay for glucose oxidase activity (GODU)

Glucose oxidase oxidizes glucose in the presence of oxygen to form gluconolactone and hydrogen peroxide. This hydrogen peroxide oxidizes ABTS-R (2,2'-azino-di-[ethyl-benzthiazoline-6-sulphonate]) in the presence of peroxidase. This generates a blue-green color which is measured using a photometer at 418 nm.

25 Reaction conditions are 90 mM glucose, 0.1 M acetate buffer, pH 5.6, incubation temperature 30°C, and reaction time 34 seconds. 1 unit (GODU) is the amount of enzyme which produces 1  $\mu$ mole hydrogen peroxide per minute at these conditions.

### Protein disulfide isomerase (PDI)

The PDI may particularly have optimum activity at pH 4 - 5.5.

30 The PDI may be from a mammalian or microbial source, e.g. from yeast or filamentous fungi, such as *Aspergillus*, *Saccharomyces* or *Schizosaccharomyces*, particularly *A. oryzae*, *A. niger*, *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*.

The PDI will typically be added in an amount of 0.2-50, particularly 1-10 mg/kg flour (calculated as pure enzyme protein).

### Dough

The dough generally comprises wheat meal or wheat flour and/or other types of meal, flour or starch such as corn flour, corn starch, rye meal, rye flour, oat flour, oat meal, soy flour, sorghum meal, sorghum flour, potato meal, potato flour or potato starch.

The dough may be fresh, frozen or par-baked.

The dough is typically leavened e.g. by adding chemical leavening agents or yeast, usually *Saccharomyces cerevisiae* (baker's yeast).

10 The dough may be a laminated dough.

The dough may also comprise other conventional dough ingredients, e.g.: proteins, such as milk powder, gluten, and soy; eggs (either whole eggs, egg yolks or egg whites); an oxidant such as ascorbic acid, potassium bromate, potassium iodate, azodicarbonamide (ADA) or ammonium persulfate; an amino acid such as L-cysteine; a sugar; a salt such as sodium chloride, calcium acetate, sodium sulfate or calcium sulfate. The dough may comprise fat (triglyceride) such as granulated fat or shortening. The dough may further comprise an emulsifier such as a monoglyceride.

### Baked product

The process of the invention may be used for any kind of baked product prepared from dough, either of a soft or a crisp character, either of a white, light or dark type. Examples are bread (in particular white, whole-meal or rye bread), typically in the form of loaves or rolls, French baguette-type bread, pita bread, tortillas, cakes, pancakes, biscuits, cookies, pie crusts, crisp bread, steamed bread, pizza and the like.

### Baking composition

25 The baking composition comprises an oxidase, a PDI and optionally an additional enzyme as described below.

The baking composition may be an enzyme preparation, e.g. in the form of a granulate or agglomerated powder. It may have a narrow particle size distribution with more than 95 % (by weight) of the particles in the range from 25 to 500  $\mu\text{m}$ . Granulates and agglomerated powders may be prepared by conventional methods, e.g. by spraying the amylase onto a carrier in a fluid-bed granulator. The carrier may consist of particulate cores having a suitable particle size. The carrier may be soluble or insoluble, e.g. a salt (such as NaCl or sodium sulfate),

a sugar (such as sucrose or lactose), a sugar alcohol (such as sorbitol), starch, rice, corn grits, or soy.

The baking composition may, in addition to enzymes, comprise other baking ingredients, particularly flour. Thus, the composition may be a dough or a flour pre-mix.

## 5 Additional enzyme

Optionally, an additional enzyme may be used together with the PDI and the oxidase.

The additional enzyme may be an amylase, a cyclodextrin glucanotransferase, a peptidase, in particular an exopeptidase, a transglutaminase, a lipase, a phospholipase, a cellulase, a hemicellulase, a protease, a glycosyltransferase, a branching enzyme (1,4- $\alpha$ -glucan  
10 branching enzyme) or a second oxidoreductase (in addition to the hydrogen peroxide-forming oxidase).

The additional enzyme may be of any origin, including mammalian and plant, and preferably of microbial (bacterial, yeast or fungal) origin.

The amylase may be fungal or bacterial, e.g. a maltogenic alpha-amylase from *B.*  
15 *stearothermophilus* or an alpha-amylase from *Bacillus*, e.g. *B. licheniformis* or *B. amyloliquefaciens*, a beta-amylase, e.g. from plant (e.g. soy bean) or from microbial sources (e.g. *Bacillus*), a glucoamylase, e.g. from *A. niger*, or a fungal alpha-amylase, e.g. from *A. oryzae*.

The hemicellulase may be a pentosanase, e.g. a xylanase which may be of microbial origin, e.g. derived from a bacterium or fungus, such as a strain of *Aspergillus*, in particular of *A.*  
20 *aculeatus*, *A. niger*, *A. awamori*, or *A. tubigensis*, from a strain of *Trichoderma*, e.g. *T. reesei*, or from a strain of *Humicola*, e.g. *H. insolens*.

The protease may be from *Bacillus*, e.g. *B. amyloliquefaciens*.

The lipase may be derived from a strain of *Thermomyces* (*Humicola*), *Rhizomucor*, *Candida*, *Aspergillus*, *Rhizopus*, or *Pseudomonas*, in particular from *T. lanuginosus* (*H. la-*  
25 *nuginosa*), *Rhizomucor miehei*, *C. antarctica*, *A. niger*, *Rhizopus delemar*, *Rhizopus arrhizus* or *P. cepacia*.

The phospholipase may have phospholipase A1 or A2 or lysophospholipase activity; it may or may not have lipase activity. It may be of animal origin, e.g. from pancreas, snake venom or bee venom, or it may be of microbial origin, e.g. from filamentous fungi, yeast or bac-  
30 teria, such as *Aspergillus* or *Fusarium*, e.g. *A. niger*, *A. oryzae* or *F. oxysporum*. Also, the variants described in WO 0032758 may be used.

The second oxidoreductase may be a peroxidase, a laccase or a lipoxxygenase.

## EXAMPLES

## Example 1

Bread was made according to a micro scale baking system using 12 g meneba flour per dough. Ascorbic acid (30 ppm), fungal alpha-amylase (Fungamyl at 10 FAU/kg flour), and xylanase (100 FXU/kg flour) were added to each dough.

Baking were made with and without addition of PDI and glucose oxidase to the dough, as indicated below. The glucose oxidase was from *Aspergillus niger*, and the PDI was a variant derived from *Aspergillus*. The volume of each loaf was measured and is expressed as specific volume.

	PDI dosage mg enzyme prote- in/kg flour	Glucose oxidase dosage GODU/ kg flour	Specific volume ml/g
Control	0	0	3.6 ± 0.2
Reference	2.5	0	3.3 ± 0.1
	5	0	3.8 ± 0.1
	0	200	3.8 ± 0.2
	2.5	200	4.0 ± 0.1
Invention	5	200	4.3 ± 0.1

The results show that the volume improvement obtained with the combination of glucose oxidase and PDI compared to the control was higher than the sum of the volume improvement obtained with either of the enzymes alone.

## CLAIMS

1. A process of preparing a baked product comprising:
  - a) adding to a dough:
    - i) a hydrogen peroxide-forming oxidase except the following:
      - 5 a) a glucose oxidase having optimum activity at pH 6-7 and having more than 75 % of maximum activity at pH 8, and
      - b) a carbohydrate oxidase which has a higher activity on an oligo-saccharide having a degree of polymerization of 2 or higher as a substrate than on the corresponding monosaccharide,
    - 10 ii) and a protein disulfide isomerase, and
  - b) baking the dough.
2. The process of the preceding claim wherein the oxidase has optimum activity at pH 4 - 5.5.
3. The process of either preceding claim wherein the oxidase is a glucose oxidase, particularly one derived from *Aspergillus niger*.  
15
4. The process of any preceding claim wherein the protein disulfide isomerase is derived from *Aspergillus*, particularly *A. niger* or *A. oryzae*.
5. A baking composition comprising:
  - a) a hydrogen peroxide-forming oxidase except the following:
    - 20 i) a glucose oxidase having optimum activity at pH 6-7 and having more than 75 % of maximum activity at pH 8, and
    - ii) a carbohydrate oxidase which has a higher activity on an oligosaccharide having a degree of polymerization of 2 or higher as a substrate than on the corresponding monosaccharide,
  - 25 b) and a protein disulfide isomerase.
6. The baking composition of the preceding claim wherein the oxidase has optimum activity at pH 4 - 5.5.
7. The baking composition of claim 5 or 6 which further comprises flour.

8. The baking composition of the preceding claim which is a dough, a flour composition, or a flour pre-mix.
9. The baking composition of any of claims 5-8 wherein the oxidase is a glucose oxidase, particularly one derived from *Aspergillus niger*.
- 5 10. The baking composition of any of claims 5-9 wherein the protein disulfide isomerase is derived from *Aspergillus*, particularly *A. niger* or *A. oryzae*.



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 01/00646

## A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A21D 8/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A21D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9957986 A1 (NOVO NORDISK BIOTECH, INC.), 18 November 1999 (18.11.99), claim 31 --	1,3-5,7-10
X	WO 9931990 A1 (NOVO NORDISK A/S), 1 July 1999 (01.07.99), page 1, line 1 - page 7, line 10, abstract --	1,3-5,7-10
X	WO 9529996 A1 (NOVO NORDISK A/S), 9 November 1995 (09.11.95) --	1,3-5,7-10

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

12 February 2002

Date of mailing of the international search report

03.04.2002

Name and mailing address of the International Searching Authority  
European Patent Office P.B. 5618 Patentlaan 2  
NL-2280 HV Rijswijk  
Tel: (+31-70)340-2040, Tx 31 651 apo nl,  
Fax: (+31-70)340-3016

Authorized officer

MOA EMLING GRÖNKVIST/BS  
Telephone No.

**INTERNATIONAL SEARCH REPORT**

International application No.

**PCT/DK 01/00646****C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9501727 A1 (QUEST INTERNATIONAL B.V.), 19 January 1995 (19.01.95), claim 11  -- -----	1

# INTERNATIONAL SEARCH REPORT

Information on patent family members

28/01/02

International application No.

PCT/DK 01/00646

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
WO	9957986	A1	18/11/99	AU	3988499 A	29/11/99
				EP	1077609 A	28/02/01
				US	6306445 B	23/10/01
WO	9931990	A1	01/07/99	AU	1751899 A	12/07/99
				CN	1283082 T	07/02/01
				EP	1041890 A	11/10/00
				US	6165761 A	26/12/00
WO	9529996	A1	09/11/95	AU	695391 B	13/08/98
				AU	2445095 A	29/11/95
				CA	2189542 A	09/11/95
				EP	0758377 A	19/02/97
				US	5741688 A	21/04/98
				US	5834280 A	10/11/98
WO	9501727	A1	19/01/95	AU	6926794 A	06/02/95